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Identification of HIV-1 Determinants for Replication *in Vivo*

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Pathogenic organisms are frequently attenuated after long-term culture *in vitro.* The mechanisms of the attenuation process are not clear, but probably involve mutations of functions required for replication and pathogenicity *in vivo.* To identify these functions, a direct comparison must be made between attenuated genomes and those that remain pathogenic *in vivo.* In this study, we used the heterochimeric SCID-hu Thy/Liv mouse as an *in vivo* model to define human immunodeficiency virus type 1 (HIV-1) determinants which are uniquely required for replication *in vivo.* The Lai/IIIB isolate and its associated infectious molecular clones (e.g., HXB2) were found to infect T cell lines but failed to replicate in the SCID-hu Thy/Liv model. When a lab worker was accidentally infected by Lai/IIIB, however, HIV-1 was isolated only from infection of primary PBMC, and not from infection of T cell lines. We hypothesized that the lab worker was exposed to a heterogeneous viral stock which had been attenuated by passage in immortalized T cell lines. Either a rare family member from this stock was selected for *in vivo* replication or, alternatively, an attenuated genotype dominant *in vitro* may have reverted to become more infectious *in vivo.* To address this hypothesis, we have used the SCID-hu Thy/Liv model to study the replication of HXB2 and of HXB2 recombinant viruses with HIV-1 fragments isolated from the infected lab worker. HXB2 showed no or very low levels of replication in the Thy/Liv organ. Replacement of its subgenomic fragment encoding the envelope gene with a corresponding fragment from the lab worker isolate generated a recombinant virus (HXB2/LW) which replicated actively in SCID-hu mice. The NEF mutation in the HXB2 genome is still present in HXB2/LW. Thus, the LW sequences encode HIV-1 determinants which enhance HIV replication *in vivo* in a NEF-independent mechanism. The specific determinants have been mapped to the V1–V3 regions of the HIV-1 genome. Six unique mutations in the V3 loop region of HXB2/LW have been identified which contribute to the increased replication *in vivo.*

INTRODUCTION

HIV-1 can infect diverse cell types *in vivo,* including $CD4^+$ T cells, macrophages, dendritic cells, Langerhans cells, and hematopoietic progenitor cells (Fauci, 1993; Levy, 1993; McCune, 1991; Weiss, 1993). HIV-1 isolates used in most studies have been expanded and maintained in immortalized human T cell lines. Since the reverse transcription process is associated with a high intrinsic error rate, it is predictable that the different selective pressures *in vitro* may lead to the generation of variants with uncertain relationship to isolates which predominate *in vivo.* Indeed, many lab-adapted isolates of HIV-1 show defects in gene functions such as vpr, vpu, and nef (Shaw *et al.,* 1984).

A particularly instructive example of such adaptation *in vitro* may be found in the passage history of HTLV-IIIB (Lai/IIIB; Chang *et al.,* 1993). Initially derived from human patient blood sample and cultured in M2T−/B cells, Lai/IIIB stock was prepared by infecting the human T leukemia cell line, H9, with infected M2T−/B cell supernatant. Subsequent analyses of HIV-1 genome from Lai/IIIB isolates showed that multiple changes have accumulated during their expansion *in vitro* (Shaw *et al.,* 1984). For example, the HXB2 genome cloned from Lai/IIIB carries mutations which lead to premature termination of three of the nine ORFs (vpr, vpu, and nef). Many other subtle mutations may have also accumulated. These mutations do not usually affect HIV-1 replication *in vitro* under the specific culture conditions. Some of them may actually enhance their replication in certain cell lines. For instance, infection of T cells *in vitro* often leads to selection of HIV-1 mutants carrying mutations in the vpr gene because vpr causes cell cycle arrest in the infected cells (Jowett *et al.,* 1995; Rogel *et al.,* 1995). However, due to lack of relevant *in vivo* models, it is not clear which mutations may contribute to HIV replication and pathogenesis *in vivo.*

Comparison of "attenuated" HIV-1 isolates with "pathogenic" ones *in vitro* and *in vivo* will help to identify important viral determinants for replication and pathogenesis *in vivo.* Two human/murine chimeric

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models have been established as *in vivo* models for studying HIV-1 replication and pathogenesis. Human peripheral blood leukocyte (PBL) cells or human fetal tissues implanted in the C.B17 scid/scid mouse (hu-PBL-SCID or SCID-hu Thy/Liv, respectively) have been successfully used to study HIV-1 replication and $CD4^+$ T cell killing *in vivo* (McCune *et al.,* 1991; Mosier *et al.,* 1989). Important, some unexpected, findings have been documented from these studies. For example, it has been shown in the hu-PBL-SCID model that a macrophage-tropic HIV-1 isolate, which is noncytopathic *in vitro,* is more potent in killing the $CD4^+$ T cells engrafted in the SCID mouse than the T-tropic (cytopathic *in vitro*) HIV-1 isolate (Mosier *et al.,* 1993). However, the human PBLs engrafted in SCID mice are physiologically abnormal due to severe xenoactivation (hyperactivated and anergic) and lack of human lymphoid organs (Huppes *et al.,* 1992; Murphy *et al.,* 1992; Tary-Lehmann and Saxon, 1992).

The SCID-hu Thy/Liv model transplants human fetal liver and thymus fragments into SCID mice (McCune *et al.,* 1991, 1988). The Thy/Liv organ developed in the mouse is tolerized to the host and supports long-term, normal T cell development (Krowka *et al.,* 1991; Namikawa *et al.,* 1990). Studies of HIV-1 infection in this model have shown that HIV-1 viruses isolated from late-stage AIDS patients replicate efficiently and lead to thymus depletion, whereas HIV-1 viruses isolated from the same patients before AIDS development replicated poorly and did not cause severe thymus deletion (Kaneshima *et al.,* 1994). As shown in the SIV/monkey model system, the HIV-1 nef function is also required for efficient replication and pathogenesis in the SCID-hu Thy/Liv model (Jamieson *et al.,* 1994). Analyses of HIV-1 recombinants bearing mutations in the accessory genes have indicated that the vif and vpu functions may also be required for this process (Aldrovandi and Zack, 1996). These HIV-1 recombinants, except the vif mutant, appeared to replicate to similar levels in PBMC. Thus, the SCID-hu Thy/Liv mouse is a relevant model to study HIV-1 replication in a human lymphoid organ.

The Lai/IIIB isolate and its associated infectious molecular clones (e.g., HXB2) were found to infect T cell lines such as H9 and to be cytopathic *in vitro* (Hahn *et al.,* 1984; Popovic *et al.,* 1984). When a lab worker was accidentally infected by Lai/IIIB, however, HIV-1 was isolated only from infection of primary PBMC, but not from infection of T cell lines (Kong *et al.,* 1989; Weiss *et al.,* 1988). We hypothesized that the lab worker was exposed to a heterogeneous viral stock which had been attenuated by passage in immortalized T cell lines. Either a rare family member from this stock was selected for *in vivo* replication or, alternatively, an attenuated genotype dominant *in vitro* may have reverted to become more infectious *in vivo.*

To address this hypothesis, we have used the SCID-hu Thy/Liv model to study the replication of HXB2 and of HXB2 recombinant viruses with HIV-1 fragments isolated from the infected lab worker (Kong *et al.,* 1989). HXB2 showed no or very low levels of replication in the Thy/Liv organ. Replacement of its subgenomic fragment encoding the envelope ORF with a corresponding fragment from the LW87-1 isolate generated a recombinant virus (HXB2/LW) which replicated actively in SCID-hu mice or in the thymus organ culture (TOC) model (Bonyhadi *et al.,* 1995). The specific determinants have been mapped to the V1–V3 regions of the HIV-1 genome. Six unique mutations in the V3 loop region have been identified which contribute to its increased replication *in vivo.*

RESULTS

Lab-adapted HIV-1 isolates acquired ability to replicate in the SCID-hu Thy/Liv mouse after passages *in vivo*

The HIV-1 clone HXB2, like its parental isolate Lai/IIIB, replicates poorly in the Thy/Liv organ (Bonyhadi *et al.,* 1995; Fig. 1C). HIV-1 isolated from the lab worker infected with IIIB/Lai showed altered tropism (Kong *et al.,* 1989; Weiss *et al.,* 1988). To characterize the mutations accumulated during *in vivo* replication, a recombinant viral genome (HXB2/LW) was generated between the molecular clone HXB2 and a 2.7-kb (*Sal*I–*Bam*HI) HIV-1 genomic fragment (LW87-1; Reitz *et al.,* 1994) obtained by short-term culture of phytohemagglutinin-activated peripheral blood mononuclear cells (PHA-PBMC) of the infected lab worker (Fig. 1A). This DNA fragment contains HIV-1 3′-half sequences encoding tat, rev, vpu, and the majority of env.

Both HXB2 and HXB2/LW replicated efficiently in PHA-PBMCs when p24 antigen levels and $TCID_{50}$ were measured (Fig. 1B). HXB2 or HXB2/LW viral supernatants were injected intrathymically (about 200 $TCID_{50}$ units/graft). HIV-1 replication was assessed in the SCID-hu Thy/Liv implant by DNA PCR analysis and p24 antigen ELISA. No significant HXB2 replication was detected until late time postinoculation (8 weeks p.i.). In contrast, challenge with the recombinant HXB2/LW virus was associated with high levels of viral replication at early time points (3–6 weeks postinoculation). Quantitation of proviral DNA confirmed that about 10% of human thymocytes were infected by HXB2/LW (Fig. 1D), comparable to the infection levels of NL4.3 or primary isolates (Su *et al.,* 1995). Thymocytes from HXB2-inoculated human thymus grafts (HB-a and HB-b; Fig. 1D) showed no sign of HIV-1 replication. The HIV gag band in lane 2 of HB-a was due to contamination, as reanalysis of the same sample showed no HIV-specific PCR product. The human β-globin product was included as a qualitative control

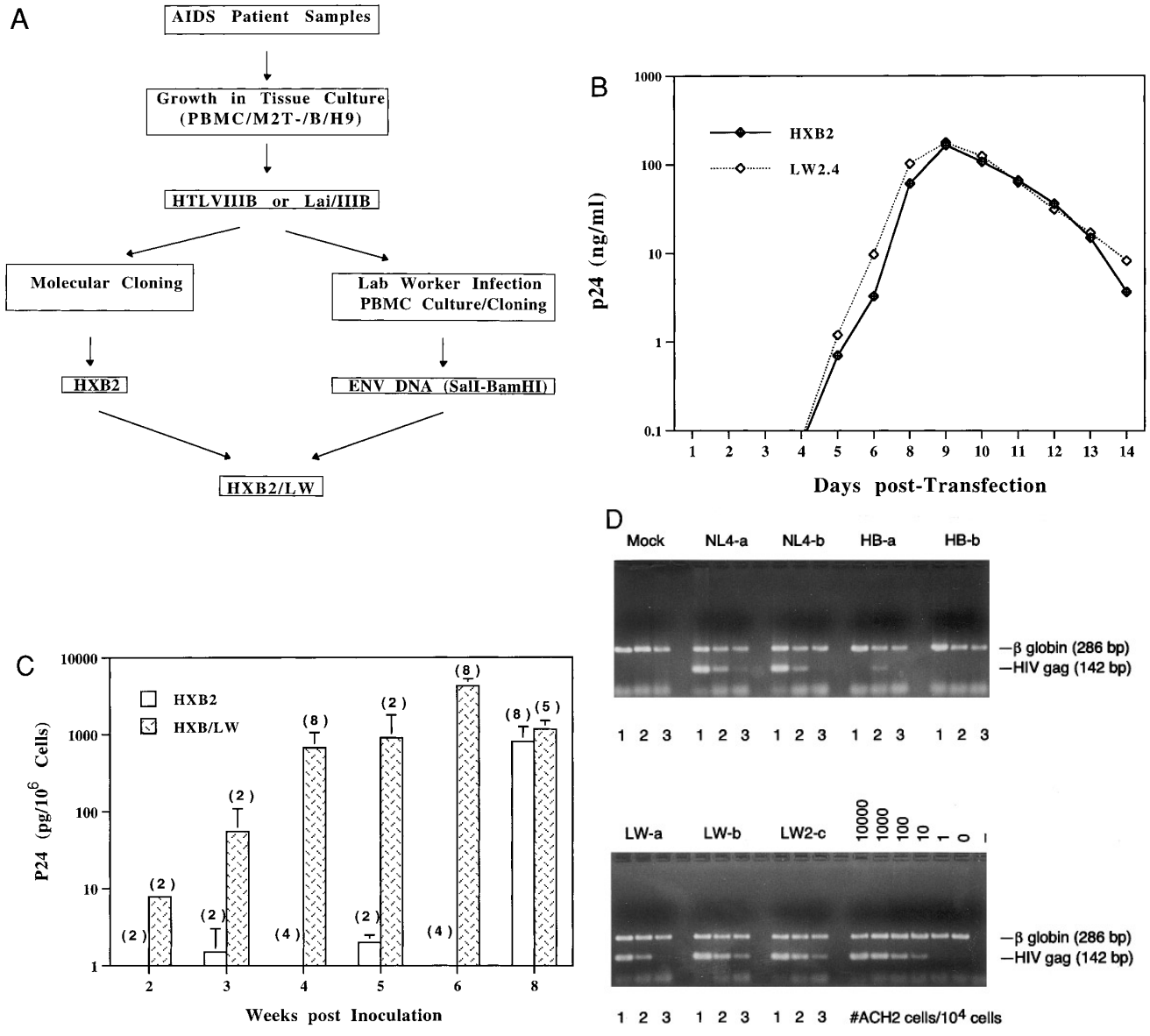

FIG. 1. (A) History of HXB2 and LW87.1, and construction of HXB2/LW. HIV-1 viral DNA sequences (*Sal*I–*Bam*HI, 5786–8466 in LW87-1, 23) from the infected lab worker were cloned to replace the *Sal*I–*Bam*HI fragment in HXB2. The resultant proviral DNA (HXB2/LW) was transfected into PHA-PBMC to generate HIV-1 viral stocks. (B) HXB2 and HXB2/LW replication in PHA-PBMC. The replication of HXB2 and HXB2/LW was compared after transfection of proviral DNA into PHA-PBMC. Supernatant p24 antigen was measured as ng/ml. Data are representative of three independent experiments. (C) HXB2 and HXB2/LW replication in SCID-hu Thy/Liv mice. The replication of HXB2 and HXB2/LW was compared in the SCID-hu Thy/Liv mouse. Cell-associated p24 antigen was measured as pg/10^6 thymocytes. Numbers in parentheses indicate number of animals analyzed. Standard errors are shown. (D) Semiquantitative PCR of infected SCID-hu thymocytes. Human thymocytes from Thy/Liv grafts infected with NL4.3 (NL4), HXB2 (HB), or HXB2/LW (LW) were assayed by semiquantitative DNA PCR. a, b, and c indicate different SCID-hu Thy/Liv mice analyzed. Lanes 1, 10,000 sample cells; lanes 2, 1000 sample cells plus 9000 normal human cells; lanes 3, 100 sample cells plus 9900 normal human cells. ACH2 cells (one HIV-1 genome/cell) were used as a standard control. –, no DNA control.

for human cells. Immunohistochemical analysis with anti-HIV-1 anti-serum demonstrated HXB2/LW-infected cells with both thymocyte and nonthymocyte morphology (data not shown). Similar results were obtained after challenge of the TOC model system (Bonyhadi *et al.,* 1995): as measured by viral p24 production, HXB2/LW but not HXB2 showed high levels of replication (Fig. 2B).

Multiple determinants contribute to the enhanced replication in the Thy/Liv organ

To map molecular determinants associated with replication *in vivo,* recombinants between HXB2 and different parts of the LW87-1 region in HXB2/LW were prepared (Fig. 2A). All of the recombinants showed similar replication capacity in PHA-PBMC except LW-H/B, which

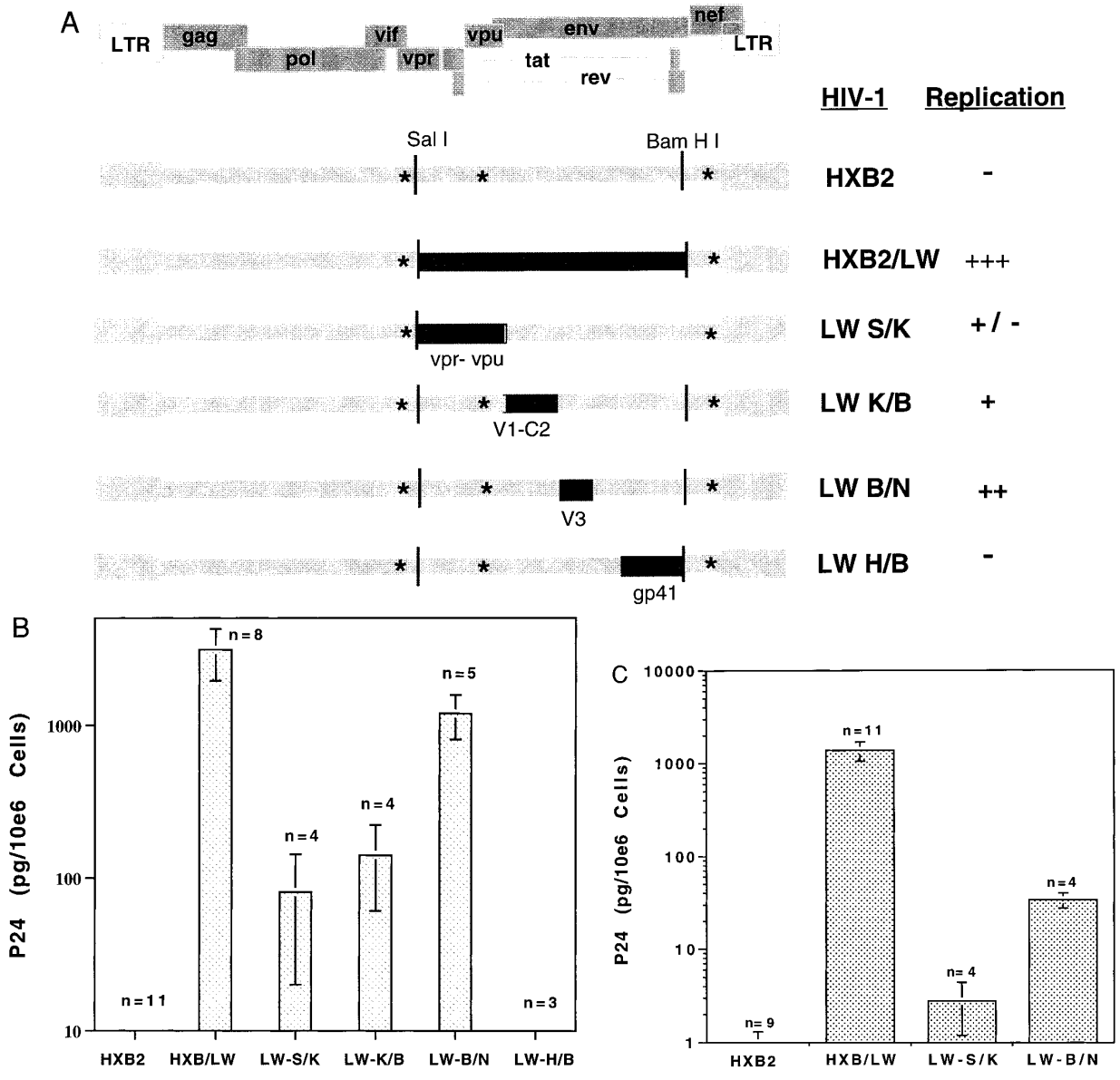

FIG. 2. Mapping of HIV-1 determinants in the LW sequences responsible for replication *in vivo.* (A) The 2.7-kb (*Sal*I to *Bam*HI) LW87-1 sequences in HXB2/LW were dissected to generate the following recombinants in the HXB2 genetic background. HXB2/LW, *Sal*I (5786) to *Bam*HI (8466); LW-S/K, *Sal*I (5786) to *Kpn*I (6348); LW-K/B, *Kpn*I (6348) to *Bgl*II (7032); LW-B/N, *Bgl*II (7032) to *Nhe*I (7257); LW-H/B, *Hin*dIII (8132) to *Bam*HI (8466). The asterisks indicate the nonsense mutations in the HXB2 genome. LW-S/K replaces the C-terminus of vpr without affecting the nonsense mutation in the vpr gene. The relative replication capacity *in vivo* (TOC and SCID-hu mice, B and C) of these recombinants is summarized on the right. +++, replication comparable to primary isolates or NL4.3 (p24 levels $\geqslant$500 pg/10^6 cells, $\geqslant$10% infection as determined by DNA PCR); ++, p24 level $\geqslant$100 pg/10^6 cells, $\geqslant$1% infection by PCR assay; +, p24 level $\geqslant$10 pg/10^6 cells, $<$1% infection by PCR assay; −, no detectable p24 production, no infection detected by PCR. (B) Replication of HXB2/LW recombinants in TOC. The TOC model was used to screen the recombinants as an initial test of their replication potentials in human thymus. p24 assays were performed 7–8 days postinfection. The labels are as in Fig. 1C. (C) Replication of HXB2/LW recombinants in SCID-hu Thy/Liv mice. HIV-1-infected SCID-hu mice were terminated and analyzed 4–6 weeks postinfection with the indicated HIV-1 clones. Labels are as described in Fig. 1C.

showed a delayed replication kinetics (data not shown). The TOC model was used to facilitate the screening of these recombinants. As shown before (Fig. 2B and Bonyhadi *et al.,* 1995), HXB2 replicated poorly in TOC. HXB2/LW, on the other hand, replicated efficiently in TOC (Fig. 2B). In SCID-hu Thy/Liv (Figs. 1C and 2C), HXB2 replicated poorly and HXB2/LW replicated to high levels. Among those recombinants which subdivided the LW87-1 region, LW-B/N (a 225-bp fragment encoding the V3 loop and some flanking env sequences) showed the highest level of replication, whereas LW-K/B (encoding V1V2C2) and LW-S/K (encoding the first exons of tat and rev, vpu, and the N-terminal 43 amino acids of env) showed lower, but significant, levels of replication. LW-H/B (encoding the second exon of tat, part of the second exon of rev, and the transmembrane portion of gp41)

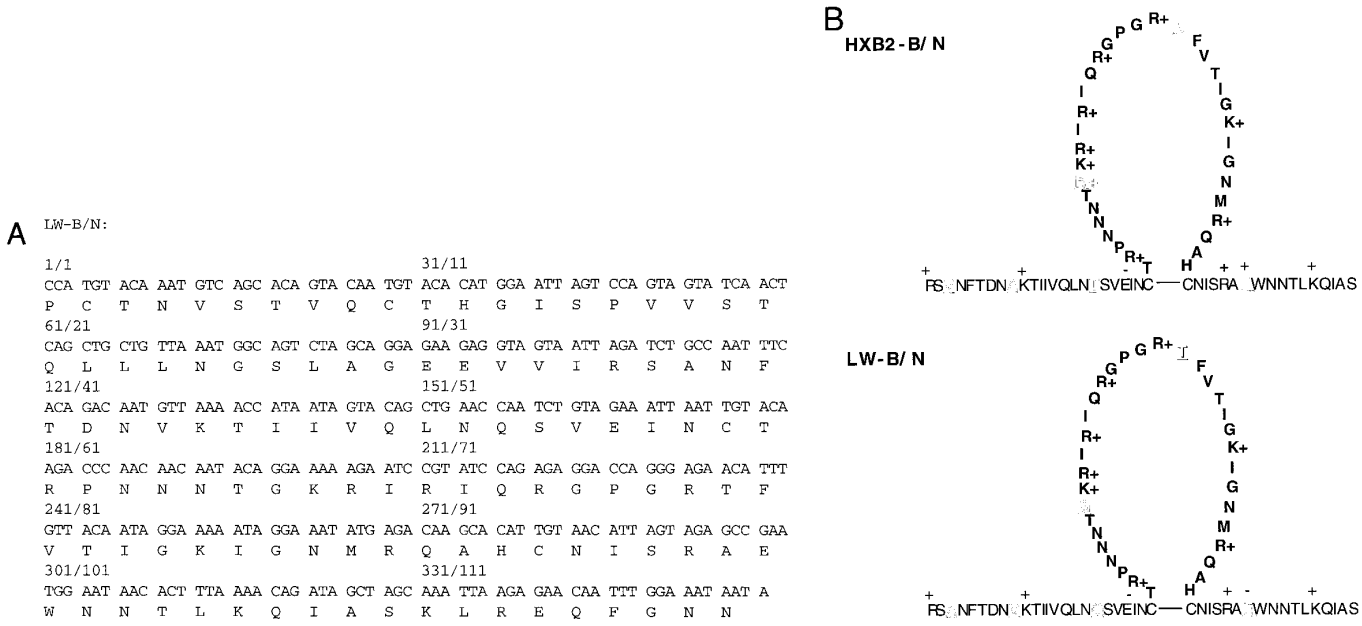

FIG. 3. LW-B/N sequence analysis. (A) DNA sequences and deduced amino acid sequences. Plasmid DNA was used directly for sequencing with primers based on HXB2 sequences before the *Bgl*II site or after the *Nhe*I site. (B) Alignment of LW and HXB2 sequences (aa) of the *Bgl*II–*Nhe*I fragment. The V3 loop is in bold and the changed residues are underlined. The charged residues are marked with + or −.

failed to show any significant replication. Notably, HXB2/LW replicated to higher levels than any of these recombinants, suggesting additive or synergistic contributions from individual, segregated regions. Figure 2A summarizes data from TOC as well as SCID-hu mice. At the resolution of this experiment, however, most of the determinants for HXB2/LW infectivity *in vivo* were carried in the V1–V3 region of env, especially in the *Bgl*II–*Nhe*I (V3 loop) fragment.

Identification of amino acid residues in the V3 loop region which contribute to the enhanced replication in the Thy/Liv organ

The LW-B/N fragment was sequenced (Fig. 3A). Comparison of its deduced amino acid sequences with that of HXB2 (Fig. 3B) revealed six amino acid changes (underlined), three N-terminal to the V3 loop, two in the V3 loop, and one immediately C-terminal to the V3 loop. The two changes in the V3 loop have been maintained in all viral isolates derived from the lab worker since 1987, 2 years after the accidental infection (Reitz *et al.,* 1994), and one, the A to T change, has been demonstrated previously to be responsible for resistance to neutralizing antibodies (Di Marzo Veronese *et al.,* 1993; Reitz *et al.,* 1994). There is otherwise no consistent correlation between the selection for (or against) these particular amino acid residues among other HIV-1 V3 loop sequences (in relation to transmission or *in vitro* growth characteristics) that have been entered in the data base (data not shown). If they are critical for infectivity of HXB2/LW *in vivo,* their role may be to affect higher order structural properties within the envelope protein. The K^+ to E^- change C-terminal to the V3 loop may also result in altered inter- or intramolecular interactions and thereby be associated with properties of infectivity *in vivo.* Further analysis of these changes may help to illuminate their relative importance to HXB2/LW replication *in vivo.*

DISCUSSION

It has been reported that HIV-1 isolates which grow out from a given patient in cell culture are not representative of those observed within the same patient *in vivo* (Meyerhans *et al.,* 1989). In the case of other lentiviruses, single point mutations have been shown to revert an attenuated to a pathogenic strain *in vivo* (Kestler *et al.,* 1991). The present study documents the first example of unique structural determinants in HIV-1 that appear to be necessary for infectivity *in vivo,* but not in PBMC or in immortalized T cell lines. Interestingly, the relevant changes do not affect the nef gene, previously implicated for pathogenicity of SIV in rhesus macaques (Kestler *et al.,* 1991) or of HIV-1 in SCID-hu mice (Jamieson *et al.,* 1994). The vpu and vpr genes, which have also been reported to affect HIV-1 replication in SCID-hu Thy/Liv mice (Aldrovandi and Zack, 1996), of HXB2/LW and the recombinants also remain defective. Thus, previously undescribed features of the V3 region of env that are necessary for infection of natural target cells are revealed by phenotypic and molecular analyses of HIV-1 isolates in the human organ implants of the SCID-hu mouse. It is noteworthy that the V3 region of NL4.3 is almost identical to that of HXB2 (only one A to V change N-terminal to

the V3 loop). It is likely that the LW env determinants bind/interact with unique target cells and/or receptors to enhance viral replication in the human thymus via a NEF-independent mechanism.

Thymocytes are clearly the primary cells infected by HXB2/LW, both by thymocyte-associated p24 assay and by immunohistochemistry (data not shown). The most likely mechanism of enhanced replication by HXB2/LW is that HXB2/LW infected certain target cells (thymocytes or macrophages) which sensitized the other thymocytes for supporting HIV-1 replication. Cytokines which enhance HIV-1 replication have been shown to be upregulated in HIV-1-infected Thy/Liv organs (M. Bonyhadi, L. Su, J. Auten, and H. Kaneshima, unpublished observations). Identification of those "initiator cells" will be of importance to understand the enhanced replication by HXB2/LW. Alternatively, the LW env region may simply interact uniquely with the receptors on human thymocytes to enhance the infectivity of HXB2/LW in human thymocytes. Identification of the target cells infected by HXB2/LW will help to clarify this question.

The replication of HXB2 in the Thy/Liv organ at late time postinfection (8 weeks p.i.; Fig. 1C) may suggest that HXB2 can infect target cells in the Thy/Liv organ. However, it either replicates or transmits very slowly in the target cells. It is also possible that HXB2 may have acquired mutations which contribute to enhanced replication at late times. It will be of interest to analyze the HIV genome from late time postinfection for possible accumulation of specific mutations (e.g., in nef and/or V3 loop regions).

It is of great interest that the same *Sal*I to *Bam*HI fragment from HXB2 also shows *in vivo* defects in the SIV–HIV chimeric (SHIV) genome (Lu *et al.,* 1996; Rei mann *et al.,* 1996). In one report, SHIV-HXB2c showed greatly reduced replication in infected monkeys compared with SHIV-89.7 (a primary HIV-1 isolate), although both chimeric viruses replicated efficiently in simian PBLs *in vitro* (Reimann *et al.,* 1996). In the second report, the SHIV-HXB2c virus appeared to be defective in establishing infection in monkeys after intravaginal inoculation (Lu *et al.,* 1996). Both reports indicate that lab-adapted HIV-1 isolates may have accumulated mutations in important genes for *in vivo* replication and transmission. However, the fragment in the SHIV chimeric viruses encodes HIV-1 vpr, vpu, rev, and tat, as well as env. It is not clear which gene(s) contributes to the observed differences.

The Thy/Liv organ of the SCID-hu mouse has served previously as a useful model for the analysis of normal thymocyte differentiation (Kraft *et al.,* 1993; Krowka *et al.,* 1991; McCune *et al.,* 1988; Vandekerckhove *et al.,* 1992; Waller *et al.,* 1992). As judged by the parameters of viral replication, tropism, cytopathic effects, and *in vivo* antiviral effects, it also appears to faithfully reproduce the expected attributes of HIV-1 infection *in vivo* (Aldrovandi *et al.,* 1993; Aldrovandi and Zack, 1996; Bonyhadi *et al.,* 1993; Jamieson *et al.,* 1994; Kaneshima *et al.,* 1994; Namikawa *et al.,* 1988; Stanley *et al.,* 1993; Su *et al.,* 1995). It is notable that HIV-1 such as HXB2 (derived from Lai/IIIB), which had been adapted in immortalized T cells and which did not grow out efficiently within the infected lab worker, also did not replicate efficiently in the SCID-hu Thy/Liv implant. Such impaired growth in the SCID-hu Thy/Liv model has been observed with an attenuated measles virus vaccine strain (Auwaerter *et al.,* 1996) and with tissue culture-adapted isolates of cytomegalovirus (Mocarski *et al.,* 1993). This small animal model may thus be useful in the definition and characterization of molecular determinants of other pathogenic organisms as well as HIV-1 which, when altered, result in attenuated growth *in vivo.*

MATERIALS AND METHODS

Construction of recombinant HIV-1 genomes

The HXB2 genomic DNA was digested with *Sal*I and *Bam*HI to drop out the 2.7-kb fragment and the corresponding fragment from LW87.1 was ligated into the provirus DNA to generate the HXB2/LW provirus. The mutations in vpr, vpu, and nef are still present in the genome.

Construction of HXB2/LW recombinant genomes was carried out by subcloning the indicated fragments in HXB2/LW into HXB2 proviral DNA. The 2.7-kb (*Sal*I to *Bam*HI) LW87-1 sequences in HXB2/LW were dissected to generate the following recombinants in the HXB2 genetic background. HXB2/LW, *Sal*I (5786) to *Bam*HI (8466); LW-S/K, *Sal*I (5786) to *Kpn*I (6348); LW-K/B, *Kpn*I (6348) to *Bgl*II (7032); LW-B/N, *Bgl*II (7032) to *Nhe*I (7257); LW-H/B, *Hin*dIII (8132) to *Bam*HI (8466). The asterisks (Fig. 2A) indicate the nonsense mutations in the HXB2 genome. LW-S/K replaces the C-terminus of vpr without affecting the nonsense mutation in the vpr gene.

HIV-1 replication in PBMC and viral supernatant production

Equal amounts of proviral DNA (20 μg) were transfected into PHA-activated PBMC as described (Su *et al.,* 1995). PHA-activated PBMC (obtained as buffy coats from Stanford Blood Center, CA) were cultured at 10^6 cells/ml with IL2 (20 units/ml). Supernatant was collected at indicated times and stored for p24 assay. $TCID_{50}$ assay (10-fold dilution) was performed with PHA-PMBC. The supernatant with $TCID_{50}$ greater than 4000/ml (p24 levels 100–200 ng/ml) was stored as viral stock for infection.

Infection of SCID-hu mice or TOC

Animal transplantation procedures for SCID-hu Thy/Liv construction have been described elsewhere (Namikawa *et al.,* 1990). Infection of SCID-Thy/Liv mice was performed as described (Su *et al.,* 1995). Briefly, SCID-

hu Thy/Liv mice (4–6 months after transplantation) were infected with supernatant collected from PHA-activated PBMC containing no HIV-1 (mock) or 4×10^3 to 10^4 $TCID_{50}$/ml of HIV-1. Fifty microliters (200 to 2000 $TCID_{50}$) were injected into each thymus graft. The Thy/Liv organs were harvested at indicated times and thymocytes were analyzed for p24 and proviral DNA.

The TOC procedures are essentially as described (Bonyhadi *et al.,* 1995). Briefly, human fetal thymuses (18–24 gestational weeks) were dissected into $\sim$1-mm^3 fragments under the dissecting scope using sterile techniques. These fragments, usually containing at least one to two intact thymic lobules, were transferred into sterile vials containing either control supernatant from mock-infected human PHA-PBMC or virus-containing supernatant from infected PHA-PBMC. The vials were gently rocked at room temperature for 2 hr, and then fragments were transferred to 0.45-μm nucleopore filters (Millipore) atop gelfoam boats (Upjohn) saturated in media (RPMI, 10% fetal calf serum, 50 μg/ml streptomycin, 50 U/ml penicillin G, 1$\times$ MEM vitamin solution [GIBCO/BRL], 1$\times$ insulin/transferrin/sodium selenite media supplement [Sigma]), in six-well tissue culture plates. The fragments were then cultured at 37°, 5% CO_2 for 7–8 days with daily changes of culture media.

Measurement of cell-associated p24 production (pg/10^6 thymocytes) was performed using a p24 ELISA kit (DuPont). Semiquantitative DNA PCR analysis was performed as described (Su *et al.,* 1995). Briefly, human thymocytes from Thy/Liv grafts infected with NL4.3, HXB2, or HXB2/LW were assayed by 10-fold dilution of infected cells into uninfected human cells. Genomic DNA was prepared from the mixed cells (Fig. 1D). Lanes 1, 10,000 sample cells; lanes 2, 1000 sample cells plus 9000 normal human cells; lanes 3, 100 sample cells plus 9900 normal human cells. ACH2 cells (one HIV-1 genome/cell) were used as a standard control.

DNA sequence analyses

The HXB2/LW-B/N proviral DNA was used directly as template to sequence with primers from either side of the insert based on sequences from HXB2. Sequences were confirmed from both strands and analyzed with the DNA Strider program.

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